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TITLE OF THE INVENTION (280 characters max)

Characterization of a Novel Receptor Kinase From Brassica with a Putative Role in Plant Defense

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Respectfully submitted,

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET (Small Entity)

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CHARACTERIZATION OF A NOVEL RECEPTOR KINASE FROM BRASSICA WITH A PUTATIVE ROLE IN PLANT DEFENSE

FIELD OF THE INVENTION

The present invention is directed to the characterization of a gene from *Brassica napus* the transcription of which is induced by wounding or chemical elicitors that mimic a pathogen attack. More particularly, the present invention is directed to the isolation of a cDNA clone designated PERK1 (Proline Extensin-like Receptor Kinase 1) which is proposed to encode a putative novel receptor kinase in *Brassica napus* and comprises a new class of plant receptor kinases characterized by an extracellular domain rich in proline and sharing sequence similarity to the extensin family of cell wall proteins.

BACKGROUND OF THE INVENTION

Receptor mediated signal perception and transduction in response to external stimuli are essential for growth and developmental processes of multicellular organisms (Mu et al., 1994). These extensively well characterized processes in animal systems involve receptor protein kinase molecules comprised of an extracellular signal perception domain, a hydrophobic transmembrane domain attached to an intracellular domain that possesses kinase activity (Horn et al., 1994). In general, transmembrane signaling by receptor protein kinases requires binding of an appropriate ligand to the extracellular domain which induces receptor dimerization and alters the activity of the intracellular catalytic domain. This promotes phosphorylation of specific substrates thereby initiating a protein kinase signaling cascade (Ullrich and Schlessinger, 1990). The majority of animal receptor protein kinases isolated to date contain tyrosine-specific kinase domains (Ullrich and Schlessinger, 1990), however, the transforming growth factor ß (TGF-ß) receptor (Lin et al., 1992) and the activin receptor (Dijke et al., 1993) possess kinase domains with serine/threonine phosphorylation activity.

Intracellular communication is also essential for the growth and development of higher plants. The extensive knowledge of cell surface receptor signaling in animal systems has resulted in the isolation of several genes predicted to encode putative receptor-like protein kinases (RLKs). The characterized members of the RLK family share highly homologous

catalytic domains with consensus sequences indicative of serine/threonine autophosphorylation activity, yet the extracellular domains of these receptors are very divergent (Braun and Walker, 1996). Five different classes of plant receptor-like protein kinases have therefore been identified according to amino acid sequence similarity in the extracellular domains of these genes. The first class of receptor kinases, designated the S-domain class, have distinct extracellular domains homologous to the S-locus glycoprotein (SLG) (Nasrallah and Nasrallah, 1993). S-domain receptor kinases have several distinguishing features such as ten conserved cysteine residues located proximal to the transmembrane domain in addition to other conserved residues implicated in the proper folding of the extracellular domain (Walker, 1994). Among this class of receptor kinases are the S-locus receptor kinases (SRKs) of *Brassica* expressed exclusively in reproductive tissues and implicated along with SLGs in controlling the sporophytic self-incompatibility response which normally inhibits self-pollination (US Patent 5,821,094; US Patent 5,484,905; Stein et al., 1991; Goring and Rothstein, 1992). Other receptor-like kinases of this type are represented in Arabidopsis by ARK1, ARK2, ARK3 (Tobias et al., 1992; Dwyer et al., 1994), in maize by ZmPK1 (Walker and Zhang, 1990) and by OsPK10 in rice (Zhao et al., 1994). The diversity in patterns of expression among members of the S-domain class suggests that these putative plant receptor kinases may be involved in mediating a variety of cellular signaling processes (Walker, 1994).

Another class of plant receptor kinases is the leucine-rich repeat (LRR) group which encodes proteins with extracellular domains containing 20-25 imperfect repeats of a 24 amino acid leucine-rich motif involved in peptide ligand recognition, cell adhesion and implicated in mediating protein-protein interactions (Braun and Walker, 1996; Wang et al., 1998). This class of plant receptor kinases include proteins such as CLAVATA1 (Clark et al., 1997) which is involved in regulating meristem and flower development in Arabidopsis, as well as proteins functioning in gamete development such as PRK1 of Petunia (Mu et al., 1994). The LRR class is represented in Arabidopsis by other receptor kinases such as ERECTA (Torii et al., 1996) which has been shown to be essential for proper plant and organ elongation, BRI1, a receptor involved in brassinosteroid signal transduction (Li and Chory, 1997), as well as TMK1 (Chang et al., 1992) and RLK5 (Walker, 1993) which may have more general roles in cellular signaling as suggested by their ubiquitous expression patterns in a variety of vegetative and reproductive

tissues. Xa21, another member of the LRR class, has been implicated in pathogen recognition by providing resistance in rice to *Xanthomonus oryzae* pv. *oryzae* (Song et al., 1995).

The lectin-like class of plant receptor kinases is represented only in *Arabidopsis thaliana* by Ath.lecRK1 (Hervè et al., 1996) and LRK1 (Swarup et al., 1996). The extracellular domain of these receptor kinases share sequence similarity with lectins which are known carbohydrate binding proteins and implicated in the transduction of oligosaccharide signals in plant cellular communication processes (Hervè et al., 1996).

The two remaining classes of plant receptor kinases isolated in *Arabidopsis thaliana* include proteins with extracellular domains containing epidermal growth factor (EGF)-like motifs found in many proteins involved in extracellular interactions (WAK1; Kohorn et al., 1992) as well as thaumatin-like domains homologous to PR5 proteins involved in pathogenesis (PR5K; Wang et al., 1996).

Extensins are particularly abundant proteins in plant cell walls and are very rich in proline and serine as well as in combinations of valine, tyrosine, lysine and/or histidine residues. The distinctive characteristic of dicot extensins is their repetitive $(Ser\text{-}Pro)_4$ pentapeptide blocks. Although extensins are synthesized as soluble precursors, the majority of proline residues are hydroxylated and both the hydroxylated proline as well as the serine residues of these proteins are glycosylated by post-translational modifications (Cassab, 1998). When secreted to the plant cell wall, extensins become rapidly insoluble, presumably due to the formation of covalent isodityrosine bridges (Cassab, 1998). Although extensins have been proposed to be structural cell wall proteins and important in development, they have also been directly implicated in plant defense against mechanical wounding (Shirsat et al., 1996) and pathogen attack (Corbin et al., 1987; Showalter, 1993).

Many of the inducible defense responses are not exclusive to mechanical wounding but are also initiated by pathogen attack. The similarity between responses to wounding and pathogen attack are not surprising since mechanical damage often precedes pathogen infection and conversely, mechanical damage may often result from a pathogen or insect attack (Truernit et al., 1996). Salicylic acid has been implicated in having an important role in the signal transduction pathway leading to systemic acquired resistance (SAR) (Penninckx et al., 1996). In general, plants challenged by mechanical wounding or pathogen attack induce rapid expression of genes (ie. proteinase inhibitor *(pin)* and pathogenesis related *(PR)* genes respectively) that are

expressed locally as well as systemically in unaffected parts of the plant (Yang et al., 1997). Increased levels of extensin transcripts as a result of mechanical wounding have been well established in various plants (Sauer et al., 1990; Shirsat et al., 1996).

It is an object of the present invention to isolate from the cDNA library of *Brassica napus* a receptor-like protein kinase designated PERK1 (Proline Extensin-like Receptor Kinase) which represents a new class of plant receptor kinases characterized by an extracellular domain rich in proline and sharing sequence similarity to the extensin family of cell wall proteins. It is postulated that PERK1 may be involved in the general perception and subsequent transduction of a wound and/or pathogen stimulus, ultimately triggering a plant's defense mechanisms and conferring broad protection against such stimuli.

SUMMARY OF THE INVENTION

In general, the present invention provides the characterization of an isolated DNA molecule comprising the nucleic acid sequence of SEQ ID No 1. In another embodiment, the present invention provides the characterization of an isolated DNA molecule having at least 30% homology to the nucleic acid sequence of SEQ ID No 1. In yet another embodiment, the present invention provides the characterization of an amino acid molecule having the amino acid sequence of SEQ ID No 2. . In yet another embodiment, the present invention provides the characterization of an isolated DNA molecule coding for the amino acid sequence of SEQ ID No 2. In a further embodiment, the present invention provides a cell transfected with the nucleic acid sequence of SEQ ID No 1.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features of the invention will now be described by way of example only, with reference to the accompanying drawings in which:

Figure 1 is the nucleotide (SEQ ID No 1) and deduced amino acid (SEQ ID No 2) sequence of the PERK1 cDNA.

Figure 1A: The DNA sequence corresponding to the PERK1 transcript is shown, with the predicted amino acid sequence presented as a single-letter code below the nucleotide sequence.

Numbers to the left refer to nucleotide sequence and the 5' and 3' untranslated regions are presented in lower case letters. Potential N-glycosylation sites (Asn-x-Ser/Thre) are indicated by dots above the Asn residues, and the extensin signature $(Ser\text{-}Pro)_4$ pentapeptide motif present in the extracellular domain is denoted in boldface type. The predicted membrane spanning region is marked by a solid underline. As defined by Hanks and Quinn (1991), the catalytic domain has been subdivided into 11 subdomains marked by dashed underlines and superscript roman numerals. The amino acids in redface type represent residues that are absolutely conserved, whereas greenface type represent groups of conserved amino acids. The two regions marked by double underlines represent consensus sequences common amongst serine/threonine kinases.

Figure 1B: Structural features of the PERK1 polypeptide. A Kyte hydropathy plot (Kyte and Doolittle, 1982) of the predicted amino acid sequence generated by DNAsis® software (Hitachi Software, San Bruno, CA) is shown, where increased hydrophobicity is denoted by positive values. The domains of the PERK1 protein are illustrated below. ECD, extracellular domain; TM, transmembrane domain.

Figure 2 represents the genomic DNA Southern Blot analysis of PERK1.

Genomic DNA (5µg) isolated from *Brassica napus* leaf tissue was digested with the indicated restriction enzymes, blotted and hybridized with a partial 1.5 kb PERK1 cDNA probe under varying conditions of stringency. DNA markers are indicated in kilobases.

Figure 2A: Genomic DNA gel blot analysis under low stringency conditions.

Figure 2B: Genomic DNA gel blot analysis under high stringency conditions.

Figure 3 illustrates the expression of PERK1 cDNA.

Figure 3A: RNA gel blot analysis of PERK1 transcripts in poly(A)$^+$ mRNA extracted from various *Brassica napus* tissues. The blot hybridized with a partial 1.5 kb PERK1 cDNA probe detected a full length transcript of ~2.2kb (⊳).

Figure 3B: The blot was subsequently probed with cyclophilin as an internal control for even loading (►).

Figure 3C: Ethidium bromide stain of gel indicates relatively equal amounts of poly(A)$^+$ mRNA were loaded.

Figure 4 illustrates a wound-inducible accumulation of PERK1 mRNA in *Brassica napus* leaf and stem tissue.

Figure 4A: Fully expanded leaves were wounded by punching out discs around the perimeter of the leaf blade. Total RNA was extracted at various time intervals after treatment, subjected to Northern blot analysis and probed with full length PERK1 cDNA (▷). The blot was reprobed with cyclophilin used as an internal control for even loading (▶). The graph represents the expression profile of PERK1 in response to wounding corrected against levels of cyclophilin expression. Control unwounded leaf tissue represented by 0 hr time point.

Figure 4B: Northern blot showing a time-course induction of PERK1 mRNA accumulation in wounded stem tissue. Total RNA harvested at the indicated time points was blotted and hybridized against the full length PERK1 coding sequence (▷). The cyclophilin loading control (▶) was used to normalize levels of PERK1 mRNA accumulation represented graphically. Control unwounded stem tissue represented by 0 hr time point.

Figure 5 describes the effects of 50µM methyl jasmonate (MeJA) on PERK1 mRNA accumulation in treated *Brassica napus* leaf and stem tissue.

Figure 5A: *Brassica napus* plants were thoroughly sprayed with a 50µM MeJA solution, and leaf tissue subsequently harvested at different time intervals after treatment. Total RNA prepared from treated leaf tissue was subjected to Northern blot analysis and probed with full length PERK1 cDNA (▷). Control plant (0 hr) was treated with the carrying solution minus the chemical inducer (0.1% [$^v/_v$] ethanol for MeJA). The blot was reprobed with cyclophilin used as an internal control for even loading (▶). The graph represents a corrected profile for the levels of PERK1 mRNA accumulation in response to treatment with MeJA normalized against levels of cyclophilin expression.

Figure 5B: Northern blot showing a time-course induction of PERK1 mRNA accumulation in MeJA treated stem tissue. Total RNA harvested at the indicated time points was blotted and

hybridized against the full length PERK1 coding sequence (▷). The cyclophilin loading control (▶) was used to normalize levels of PERK1 mRNA accumulation represented graphically.

Figure 6 illustrates the effects of 4mM salicylic acid (SA) on PERK1 mRNA accumulation in treated *Brassica napus* leaf and stem tissue.

Figure 6A: *Brassica napus* plants were thoroughly sprayed with a 4mM SA solution, and leaf tissue subsequently harvested at different time intervals after treatment. Total RNA prepared from treated leaf tissue was subjected to Northern blot analysis and probed with full length PERK1 cDNA (▷). Control plant (0 hr) was treated with the carrying solution minus the chemical inducer (5mM phosphate buffer, pH7). The blot was reprobed with cyclophilin used as an internal control for even loading (▶). The graph represents a corrected profile for the levels of PERK1 mRNA accumulation in response to treatment with SA normalized against levels of cyclophilin expression.

Figure 6B: Northern blot showing a time-course induction of PERK1 mRNA accumulation in SA treated stem tissue. Total RNA harvested at the indicated time points was blotted and hybridized against the full length PERK1 coding sequence (▷). The cyclophilin loading control (▶) was used to normalize levels of PERK1 mRNA accumulation represented graphically.

Figure 7 is the proposed pathway mediating PERK1 expression in response to wounding, MeJA and SA treatments.

Figure 8 is a Western blot illustrating the bacterially expressed extracellular domain of PERK1.

Figure 8A: Western blot performed on the bacterially expressed extracellular domain fusion protein using a T7 monoclonal antibody which recognizes the T7 epitope on the bacterially expressed fusion protein. There was a marked induction of the fusion protein in the presence of IPTG (Isopropyl-β-d-Thiogalactopyranoside) (Figure 8A; lane 2), however recovery of the purified protein (Lane 4 ;♦) was not very efficient. The presence of the fusion protein in the insoluble fraction (Lane 3) suggests that the extracellular domain is likely insoluble and could be purified from inclusion bodies.

Figure 8B: Western blot performed to confirm that the extracellular domain fusion protein is targeted to inclusion bodies. Induction of the fusion protein was efficient (Figure 8B; lane2), and despite its presence in the supernatant (Lane 3) and in the denatured insoluble samples (Lane 5), the fusion protein is predominantly in the insoluble fraction (IB-inclusion body) (Lane 4 ; ◊) Lanes 6-9 represent the pTric HisC negative control indicating that the signals obtained for the expression of the extracellular domain protein are specific to the fusion protein.

Figure 9 is a Western blot illustrating the bacterially expressed full length PERK1.

Western blot conducted on the bacterially expressed PERK1 protein to determine whether the full length protein is insoluble and forms inclusion bodies. Although the induction of the full length protein was not as pronounced (Figure 9; lane 2) it is quite evident that the fusion protein is predominantly localized in the insoluble fraction (ie. inclusion body) (Lane 4; ■) Lanes 6-9 represent the parental plasmid as mentioned above.

Figure 10 is a Western blot illustrating the bacterial expression and kinase assay of wild-type and mutated catalytic domains of PERK1.

Figure 10A: A Western blot was performed to confirm the induction and purification of both the wild-type (★) and mutated (◀) catalytic domain fusion proteins using an anti-MBP antibody. The wild-type fusion protein appears to be toxic in bacteria which compromises its inducibility and purification (Figure 10A; lanes 3-4). In addition, the wild-type fusion protein migrates at a slightly larger molecular weight than does the mutated protein. The mutated fusion protein is induced and purified more efficiently, perhaps due to the fact that it is no longer kinase active (Figure 10A ; Lanes 6-7). Purified pMAL-c (Lane 1; •) was used as a negative control for this experiment.

Figure 10B: A kinase assay performed on affinity purified wild-type and mutated fusion proteins incubated in the presence of γ-^{32}PdATP. Detection of a phosphoprotein only in Lane 1 provides direct biochemical evidence that the wild-type catalytic domain (★) of PERK1 encodes a functional protein kinase that is capable of autophosphorylation (Lane 1) and that the mutation successfully abolished kinase activity (Lane 2).

Figure 11 illustrates a homology of PERK1 to predicted proteins from the *Arabidopsis* genome sequencing project.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

For convenience in the following description, like numerals refer to like structures in the drawings.

The present invention is directed to an isolated gene, PERK1(Proline Extensin-like Receptor Kinase) (SEQ ID No 1, Figure 1A), which was isolated from λ-pistil cDNA library of *Brassica napus* and represents a novel class of receptor kinases in higher plants. Genomic Southern blot analysis under low and high stringency conditions revealed that PERK1 of the present invention is a single copy gene in the *Brassica* genome (Figures 2A and 2B). PERK1 of the present invention is ubiquitously expressed at high levels in root, stem, petal and pistil and less abundant in leaf tissue (Figure 3A).

In its second aspect, the present invention is directed to PERK1 which , based on its deduced amino acid sequence (SEQ ID No 2, Figure 1A), is predicted to encode a receptor-like kinase possessing an extracellular domain, a single membrane spanning domain and an intracellular kinase domain. The extracellular domain of the protein of the present invention consists of 137 amino acids (Figure 1A) rich in proline and sharing sequence similarity with extensins, a family of hydroxyproline-rich glycoproteins (HRGPs) that constitute a major protein component of higher plant cell walls (Showalter, 1993).

The present invention is further directed to the rapid accumulation of PERK1 mRNA in leaf and stem tissue of *B. napus* in response to wounding (Figure 4) and pathogen attack mimicked by salicylic acid as a chemical elicitor (Figure 6). Therefore, it was suggested that PERK1 of the present invention is a signaling molecule associated with the cell wall via its extensin-like extracellular domain and involved in the transduction of extracellular stimuli (eg. wounding, pathogen attack) into an intracellular response through a cytoplasmic kinase domain, thereby bridging the cell wall - plasma membrane continuum.

In its third aspect, the present invention is directed to the generation of a PERK1 antibody. The coding sequence for the region containing the extracellular domain of PERK1 of the present invention (Figure 8) as well as the coding sequence representing the entire protein

(Figure 9) were cloned into the pTricHis expression plasmid for production of His-tagged fusion proteins in *E. coli* and purification by affinity chromatography on a Talon resin column. The bacterially expressed catalytic domain fusion protein of PERK1 was further tested for kinase activity. The Western blot (Figure 10) confirmed that PERK1 of the present invention encodes a protein with kinase activity.

MATERIALS AND METHODS

Construction of λ-Pistil cDNA Library

Pistils were collected from floral buds of Westar and W1 cultivars 1-2 days before anthesis. Total RNA was isolated using the method described by Jones et al. (1985), and enriched for poly(A)$^+$ mRNA by affinity chromatography using pre-packed oligo $(dT)_{25}$-cellulose beads (New England Biolabs, Beverly, MA). Approximately five micrograms of pistil poly(A)$^+$ mRNA was used for the construction of a cDNA library using the ZAP-cDNA® synthesis kit (Stratagene, La Jolla, CA). The information encoded by the poly(A)$^+$ mRNA was reversed transcribed using MMLV-RT and converted into stable, unidirectional cDNA which was subsequently inserted into a self-replicating Uni-ZAP XR vector, packaged into phage particles in three separate packaging reactions and amplified as described by the manufacturer's procedures (Stratagene, La Jolla, CA). Infection of *Escherichia coli* host strain XLI-Blue yielded a primary library with an average titer of 1.0×10^6 plaque forming units. The primary library was subsequently amplified to obtain an average total of 6.6×10^{10} plaque forming units.

Generation of Putative Novel Receptor-like Protein Kinase Clones

The isolation of putative novel *Brassica napus* receptor kinases relied upon the newly constructed λ-pistil cDNA library and involved *in vivo* mass excision of the pBluecsript phagemids from the Uni-ZAP XR vectors as outlined by the manufacturer (Stratagene, La Jolla, CA). Following efficient mass excision, phagemid DNA was extracted using a large scale alkaline protocol as described by Sambrook et al. (1989) and subjected to the polymerase chain reaction (PCR) using two separate oligonucleotide combinations, RK1/RK2 and RK1/RK3 (otained from M. Cock, École Normale Supérieure de Lyon, France) specifically designed to prime conserved subdomains of the catalytic domain of receptor protein kinases. RK1 (5-ggiggTTTCggiAT^{T}c$_{A}$gTiTT^{A}$_{T}^{T}_{C}$AA^{A}$_{g}$gg - 3') served as the forward primer and was constructed based

upon a conserved amino acid consensus (GGFGIV$^{F}/_{Y}$KG) within subdomain I of the catalytic domain. In the above sequence, and other sequences in this application, the subscripts and superscripts signify variable nucleotide or amino acid residues. The degeneracy of one reverse primer RK2 (5' - AAiATiC$^{T}_{g}$igCCATiCC$^{A}_{g}$AA$^{A}_{g}$$^{T}_{C}$ - 3') reflects a conserved amino acid consensus (DFGMARIF) of subdomain VII which closely resembles the SRKs in Brassica. The second reverse oligonucleotide RK3 (5' - A$^{g}_{A}$iA$^{g}_{A}$$^{T}_{C}$TTigCiA$^{A}_{g}iCC^{A}_{g}AA^{A}_{g}$TC - 3') was generated based upon conserved amino acids (DFGLAKLL) within subdomain VII prevalent among the RLKs isolated in Arabidopsis. Phagemid DNA was amplified in a reaction mixture containing 1µl of excised phagemid DNA, 10x PCR buffer (100mM Tris-HCl pH8.3, 500mM KCl, 15mM $MgCl_2$), 10mM deoxyribonucleotide triphosphate mixture, 10µM of each oligonucleotide primer and 0.5µl Tsg polymerase (BioBasics, Canada). The PCR reaction was heated at 95°C for 2 min and amplified for 35 cycles under the following amplification conditions: 1 min at 95°C for denaturation, 1 min 30 sec at 50°C for primer annealing and 1 min at 72°C for synthesis. A final extension cycle of 10 min at 72°C was also incorporated into the amplification program. All PCR products generated of the expected size (420-450 bp) were gel purified, cloned into the pT7Blue plasmid (Novagen, Madison, WI) and introduced into *Escherichia coli* DH5α Transformants were tested for the presence of an insert and positive clones were sequenced with universal primers (R-20 and U-19) by an ABI automated sequencer (Model 373 STRETCH DNA; Perkin Elmer Corp., Canada Ltd.) using the dideoxychain-terminating method described by Sanger et al. (1977). Sequence analyses performed using DNAsis® software (Hitachi Software, San Bruno, CA) at the nucleotide and amino acid levels yielding homologies less than 85% and 70% respectively were considered novel and led to the isolation of four putative novel *B.napus* kinases, one of which is PERK1.

Screening of λ-Pistil cDNA Library

The original 351 bp PCR product was used to screen the λ-pistil cDNA library. Approximately 2x10^{6} plaques from the amplifiied library were screened and plated at a density of 1x10^{5} pfu/plate. Duplicate colony lifts were performed according to Sambrook et al. (1989), and prehybridized for 2 hr at 42°C in 50% formamide, 5x Denhardt's solution (1x Denhardt's solution is 0.02% Ficoll, 0.02% DVP, 0.02% BSA), 5x SSC (1x SSC is 0.15M NaCl, 0.015M sodium citrate), 0.1% SDS, 1mM EDTA and 100µg/ml salmon sperm DNA. Filters were

subsequently hybridized overnight in the same solution containing the 351 bp PERK1 cDNA radiolabeled by random priming (Feinberg and Vogelstein, 1983) and washed twice with 2x SSC, 0.1% SDS at room temperature for 15 min, followed by two 25 min washes with 0.5x SSC, 0.1% SDS at 55°C. Plaques containing putative positive clones were cored and subjected to several rounds of screening until single isolates representing the PERK1 clone were obtained. Single clone excision to liberate the double stranded pBluescript phagemid was performed on each isolate according to the procedure recommended by the manufacturer (Stratagene, La Jolla, CA). Phagemid DNA digested with EcoRI/XhoI to release the cloned cDNA was subjected to standard plasmid Southern blot analysis as described by Sambrook et al. (1989) and probed with the radiolabeled 351 bp PERK1 cDNA. The membrane was prehybridized at 42°C in 5x SSPE, 10x Denhardt's solution and 0.5% SDS for 2 hr and hybridized overnight at the same temperature in a buffer containing 50% formamide, 5x SSPE and 0.5% SDS. Washing conditions were performed twice at room temperature for 15 min in 2x SSC, 0.1% SDS followed by several 30 min washes at 55-60°C in 0.1x SSC, 0.1% SDS. An intense hybridization signal would confirm whether phagemids isolated from the library screen contained the cloned cDNA of interest. Several positive clones were sequenced as previously mentioned using both universal and sequence specific primers to generate a consensus sequence representing the PERK1 cDNA clone (1512bp) isolated from the λ-pistil cDNA library.

Rapid Amplification of cDNA Ends (5'RACE)

The 5' end of the PERK1 cDNA was obtained by the procedure for the rapid amplification of cDNA ends originally described by Frohman et al. (1988) using the 5' RACE System, Version 2.0 kit (Gibco-BRL, Gainthersburg, MD). First strand cDNA was synthesized from approximately 300μg of mixed Westar and W1 pistil total RNA using a gene specific primer GSP1 (5'-TAACCAACAAgAgACA-3') designed to anneal approximately 300 bp from the 5' end of the PERK1 cDNA (1512 bp) isolated from the library screen. Following cDNA synthesis, the first strand product was purified from unincorporated dNTPs and GSP1 using a GLASS MAX® spin cartridge. A homopolymeric tail was added to the 3' end of the cDNA using TdT (terminal deoxynucleotidyl transferase) and dCTP. Tailed cDNA was amplified using a second gene specific primer GSP2 (5'-CCACTCCCAACTTTCAAC-3') desgined to anneal 3' to GSP1 with respect to the cDNA, and an abridged anchor primer (Gibco-BRL, Gainthersburg, MD) which

annealed to the homopolymeric tail. PCR amplification was carried out for 35 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min and extension at 72°C for 2 min, followed by a final extension cycle for 10 min. A PCR product of the expected size (~1 kb) corresponding to the 5'end of PERK1 was gel purified, cloned into the pT7Blue plasmid (Novagen, Madison, WI) and transformed into *Escherichia coli* DH5α. Confirmation of the 5'RACE product was obtained by plasmid Southern blot analysis as described above and by sequential primer based sequencing.

Cloning of Full Length PERK1 cDNA

A PCR based approach was used to generate a full length PERK1 cDNA by combining the 5'RACE product cloned into the EcoRV site of pT7Blue with the cDNA isolated from the library screen cloned into the EcoRI/XhoI sites of the pBluescript SK phagemid. A forward primer (5'-ggAAAgCTTgCATgCCTgCAggTCgAC -3') containing an internal PstI site was designed to anneal upstream to the EcoRV cloning site of pT7Blue. A reverse primer (5'-CgCCTgCAggTAATACgACTCACTATAggg -3') also containing a PstI site was designed based on pBluecsript phagemid sequence immediately 3' to the EcoRI/XhoI cloning site. Full length PERK1 cDNA was generated from a 100µl PCR reaction containing 1µl (~20ng) of each template (cDNA in pT7Blue and pBluescript phagemid), 10x Pfu Buffer (200mM Tris-HCl pH8.8, 100mM $(NH_4)_2SO_4$, 20mM $MgSO_4$, 1% Triton®X-100, 1mg/mlBSA), 10mM dNTPs, 50pmol forward and reverse primers and 1µl Pfu polymerase (Gibco-BRL, Gainthersburg, MD). The samples were heated to 94°C for 5 min and amplified for 30 cycles with a denaturing cycle of 1 min, a primer annealing cycle at 53°C for 1 min followed by an extension cycle for 3 min at 72°C. The resulting PCR product of the expected size (~2.2kb) was gel purified and cloned into the PstI restriction site of pBluescript KS (+/-) II. The full length PERK1 cDNA sequence was confirmed by a sequential primer based sequencing approach using both universal and sequence specific primers as previously described. All DNA and protein sequence analysis was performed using the DNAsis® Software (Hitachi Software, San Bruno,CA).

Genomic DNA Isolation and Southern Blot Analysis

Genomic DNA was extracted from approximately one gram of young *Brassica napus* leaf tissue according to the method described by Goring et al. (1992b). Approximately 5µg of

genomic DNA was digested with several restriction enzymes (BamHI, EcoRI, HindIII, PstI, XbaI, XhoI), fractionated through a 0.8% agarose gel and transferred overnight in 10x SSC onto Zetaprobe membrane (Biorad, Hercules,CA). This was performed in duplicate to test hybridization conditions under low and high stringencies conditions. After drying, the membranes were prewashed in 0.1x SSC, 0.5% SDS for 25 min at 60°C. The membranes were prehybridized and hybridized as previously described for plasmid Southern blots with the inclusion of 10% dextran sulfate and 50µg/ml salmon sperm DNA in the hybridization buffer. Washing conditions for genomic southern blots varied depending on the stringency tested. One membrane was washed under conditions of low stringency for 15 min at room temperature in 2x SSC, 0.1% SDS followed by second 15 min room temperature wash in 1x SSC, 0.1% SDS and three final washes at 50°C in 1x SSC, 0.1% SDS.. The second membrane was washed under conditions of high stringency by lowering the salt concentration to 0.1x SSC, 0.1% SDS and increasing the temperature to 65°C. The ^{32}P-labeled 1512 bp PERK1 cDNA probe was generated by random priming as described by Feinberg and Vogelstein (1983). Membranes were subjected to autoradiography (XAR-5 film, Kodak) overnight at -80°C.

Isolation and Northern Blot Analysis of Multiple Tissue RNA

Total RNA was extracted from a mixture of Westar and W1 root, stem, leaf, petal, anther and pistil tissue as described by Jones et al. (1985). Poly(A)$^+$ mRNA was isolated using the polyA Spin™ mRNA Isolation kit as outlined by the manufacturer's procedure (New England Biolabs, Beverly, MA). Approximately 3µg of poly(A)$^+$ mRNA was fractionated on a 1.2% formaldehyde gel (Sambrook et al., 1989) and transferred to Zetaprobe membrane (Biorad, Hercules, CA) in 10x SSC. Hybridization and high stringency wash conditions were conducted as previously described for genomic Southern blot analysis. The membrane was subsequently probed with a cyclophilin EST clone (No. mBN086) as an internal control for even loading of poly(A)$^+$ mRNA.

Plant Treatments

B. napus plants were grown in a growth chamber at 22°C with a 16hr light period followed by an 8hr dark period at 16°C. Experiments were conducted on two month old plants, and all experiments used one plant per time point from which leaf and stem tissue was harvested.

Wounding of leaf material was performed by punching out leaf discs every 1cm around the perimeter of the leaf blade ensuring that the midvein remained intact, and stems were wounded by slicing into 1-3cm segments. The wounded tissues were placed in petri dishes containing filter paper moistened with 20mM sodium phosphate buffer supplemented with 50μg/ml chloramphenicol to prevent bacterial contamination of the wounded tissue (Shirsat et al., 1996). A control (0 hr) time point for this experiment was performed by incubating unwounded tissue in the sodium phosphate buffer. Wounded leaf and stem tissue was harvested at different times after wouding (0 hr, 5 min, 15 min, 45 min, 1 hr, 4 hr, 12 hr, 24 hr, 36 hr, 48 hr).

Plants were thoroughly sprayed with 50μM methyl jasmonate (MeJA; Sigma, St. Louis, MO) (Titarenko et al., 1997) and 4mM salicylic acid (SA; Sigma, St. Louis, MO) solutions (Schweizer et al., 1998). Leaf and stem tissue was harvested at various time points (0 hr, 5 min, 15 min, 30 min, 45 min, 1 hr, 2 hr, 4 hr, 12 hr, 24 hr, 36 hr, 48 hr, 72 hr, 96 hr) following SA and MeJA treatments. Control sprays were performed with the carrying solutions in the absence of the chemical inducer. Carrying solutions were 5mM phosphate buffer, pH 7 for SA, and 0.1% [$^{v}/_{v}$] ethanol for MeJA.

Total RNA was extracted from treated tissue according to the method described by Cock et al. (1997). Depending on the treatment, varying amounts of total RNA (20-40μg) was electrophoresed on a 1.2% formaldehyde gel and standard Northern blot analysis was performed as described by Sambrook et al. (1989). Hybridization and washing conditions were performed as outlined for the multiple tissue northern blot. Following autoradiography, the amounts of radioactive signal were quantified using Instant Imager Electronic Autoradiography (Packard, Meriden, CT). The membranes were reprobed with the cyclohpilin cDNA and the amounts of hybridized radiolabeled cyclophilin were quantified in the same manner. The relative amounts of RNA hybridized to the full length PERK1 cDNA probe were determined after correction for differences in the amounts of cyclophilin RNA.

RESULTS

Isolation and Sequence Analyses of PERK1 cDNA

In order to isolate novel receptor-like protein kinases in *B. napus* a combination of degenerate oligonucleotide primers designed against conserved kinase subdomains I and VII (Hanks and Quinn, 1991) were used to amplify mass excised phagemid DNA from a newly constructed λ-pistil cDNA library. The cDNAs encoding products of the expected length (~420-450 bp) were cloned and the deduced partial sequences were analysed against several databases in order to determine which clone represented a putative novel kinase. One of several candidates, showed the highest degree of sequence similarity to the cytoplasmic domain of known plant receptor protein kinases, and was therefore used to screen the amplified λ-pistil cDNA library. Several positive clones obtained from the library screen were completely sequenced and a partial 1512 bp consensus sequence was generated to represent the PERK1 cDNA isolated from the library screen. Although this partial PERK1 cDNA had an open reading frame, it did not encode a full length transcript, therefore the 5' end was completed by 5' RACE (see Methods).

The deduced amino acid sequence of PERK1 is shown in Figure 1A and a schematic representation of its hydropathy plot is shown in Figure 1B. The full length cDNA sequence is 2189 bp and consists of one large open reading frame of 1944 bp encoding a predicted protein of 648 amino acids with an estimated molecular mass of 69 kDa (Figure 1). The first methionine of this open reading frame is preceded by two in frame stop codons, TAA and TGA at positions -48 to -45 and -23 to -21 respectively. In addition, there is also an AGAA sequence at position -9 to -6 (Figure 1) which is a favourable site for translation initiation in all eukaryotes (Lutcke et al., 1987).

Based on this sequence, PERK1 is predicted to encode a receptor-like kinase possessing an extracellular domain, a single membrane spanning domain and an intracellular kinase domain (Figure 1B) with three potential N-linked glycosylation sites (Asn-X-Ser/Thr) found throughout the sequence (Figure 1A) (Weinstein et al., 1982). The predicted polypeptide sequence was analyzed using the PSORT database and determined to be a Type Ib intergral membrane protein with a hydrophilic amino terminal domain exposed on the exterior of the membrane but whose coding sequence does not indicate a cleavable signal sequence preceding this domain. Singer

(1990) proposes that despite the lack of a signal peptide, Type Ib integral membrane proteins are inserted into the membrane via the usual ER-translocator protein machinery with some slight modifications. The extracellular domain of this protein consists of 137 amino acids (Figure 1A) rich in proline and sharing sequence similarity with extensins, a family of hydroxyproline-rich glycoproteins (HRGPs) that constitute a major protein component of higher plant cell walls (Showalter, 1993). Extensin proteins have two proposed functions in plants, one which contributes to the structural support of the cell wall by forming glycoprotein networks and the other which involves plant defense; helping to protect the plant against mechanical wounding or pathogen attack (Wilson and Fry, 1986; Showlater, 1993). A distinctive characteristic prevalent among dicot extensins is the repetitive Ser-$(Pro)_4$ pentapeptide consensus motif (Showalter, 1993). A unique feature of PERK1's extracellular domain is the predominance of a slightly modified Ser-$(Pro)_{2-3}$ motif in addition to the presence of one signature pentapeptide block (Figure 1A). In order to investigate the phylogenetic status of PERK1, sixty four deduced amino sequences corresponding to the extracellular and transmembrane domains of extensin, proline rich and other cell wall proteins were retrieved from Genbank and used to construct a phylogenetic tree (Clustal X). Results from the phylogenetic analysis indicated that PERK1 is most similar to a subset of extensin proteins as suggested by the sequence homology restricted predominantly to the serine/proline rich regions of the protein (data not shown).

The predicted protein also contains two other domains of note. Hydropathy analysis (Kyte and Doolittle, 1982) of the protein sequence predicted a membrane spanning region of 23 amino acids (Figure 1; residues 138-160) followed by a characteristic stop transfer sequence rich in charged amino acids [Arg-Arg-Arg] required for the proper insertion in the membane (Weinstein et al., 1982).

All known protein kinases display amino acid sequence similarity in their catalytic domains which are comprised of eleven subdomains containing some invariant residues important for catalysis (Hanks and Quinn, 1991). The overall features of this organization are identified in the catalytic domain (residues 274-548) of the PERK1 protein in that all of the absolutely conserved amino acids as well as the highly conserved amino acid groups are present (Figure 1A). The sequences of DIKASN in subdomain VI and GTFGYLAPE in subdomain VIII (Hanks and Quinn, 1991) are strong indicators that PERK1 may possess serine/threonine

rather than tyrosine substrate specificity (Figure 1A) which likely suggests a role for PERK1 in plant signal transduction (Hanks and Quinn, 1991)..

PERK1 is a Single Copy Gene and Ubiquitously Expressed in *B. napus* Tissue

As shown in Figure 2, Southern blot analysis was performed under conditions of varying stringency using *B. napus* genomic DNA digested with several restriction enzymes in order to determine copy number of PERK1 in the Brassica genome. Based on known restriction sites within the cDNA and identical hybridization patterns obtained for low and high stringency conditions (Figure 2), PERK1 appears to exist as a single copy gene and is not a member of a multigene family.

In order to determine whether PERK1 is expressed in plant tissues, RNA gel blot analysis was performed using poly(A)$^+$ mRNA isolated from a variety of *B. napus* tissues as shown in Figure 3. A partial 1.5 kb PERK1 cDNA probe used in this experiment detected a transcript of 2.2 kb (Figure 3A) which is consistent with the size of the full length PERK1 cDNA. The presence of a higher molecular weight transcript of ~ 4.4 kb in root (Figure 3A) may represent an alternatively spliced transcript of PERK1 generated during mRNA processing. This is reminiscent of the alternative transcript detected for the carrot extensin gene which as reported by Chen and Varner (1985) is predominantly expressed in root tissue.

The 2.2 kb PERK1 transcript was most abundant in *B. napus* root, stem and petal tissue (Figure 3A). Levels of PERK1 mRNA were also detected in leaf and pistil tissue albeit at much lower levels and transcripts appear to be absent in the anther tissue (Figure 3A). As an internal control the blot was reprobed with a cyclophilin EST cDNA to ensure even loading of the poly(A)$^+$ mRNA. A 700 bp cyclophilin transcript detected with relatively the same intensity in all tissues with the exception of anther indicates that equal amounts of mRNA was used (Figure 3B). In addition, the ethidium bromide stained gel shows relatively even loading (Figure 3C). The striking difference in the intensity of the cyclophilin signal in anther tissue (Figure 3B) is a common problem associated with the desiccate nature of the tissue, and could be attributed to the low abundance of cyclophilin mRNA present in the anther tissue as reported by Gasser et al. (1990).

Changes is PERK1 mRNA in Response to Wounding and Chemical Elicitors

The increasing evidence that protein kinases play important roles in plant defense (Zhou et al., 1995; Usami et al., 1995; Suzuki and Shinhi, 1995), in addition to the significant homology shared between the extracellular domain of PERK1 and extensin proteins prompted us to address a possible role for PERK1 in mediating plant responses to mechanical wounding and pathogen attack. In contrast to extensin genes isolated from many other plant species including *Arabidopsis* (Merkouropolous et al., 1999), carrot (Chen and Varner, 1985), tobacco (Keller and Lamb, 1989) and tomato (Showalter et al., 1991) which are primarily comprised of tandem repeats of the extensin motif, a striking and unique feature in the organization of PERK1 is the presence of a catalytic domain. It is conceivable that PERK1 is a signaling molecule associated with the cell wall via its extensin-like extracellular domain and involved in the transduction of extracellular stimuli (eg. wounding, pathogen attack) into an intracellular response through a cytoplasmic kinase domain, thereby bridging the cell wall - plasma membrane continuum.

In order to examine whether PERK1 expression could be influenced by external stimuli, leaf and stem tissue of *B. napus* plants were wounded and the abundance of PERK1 mRNA was determined by standard Northern blot analysis using the full length PERK1 cDNA as a probe (see Methods). Figure 4 shows changes in the steady-state levels of PERK1 mRNA accumulation following injury. PERK1 transcripts in wounded leaf tissue began to accumulate 5 min after wounding, reaching maximal levels within 15 min post injury represented by an 8.2 fold induction. A 4.5 fold increase in PERK1 mRNA levels was detected 45 min following treatment declining towards basal levels by 1 hr (Figure 4A).

A similar profile of PERK1 mRNA steady state levels was obtained for wounded stem tissue (Figure 4B). An accumulation of PERK1 mRNA in stem is evident 15 min following wounding which represents a 3.6 fold induction of this gene. Maximum steady state levels of PERK1 mRNA in stem was achieved 45 min after injury corresponding to a 4.5 fold induction. Therefore, the overall kinetics of PERK1 mRNA accumulation in both tissues after mechanical wounding is clearly a very rapid response (Figure 4).

Defense mechanisms deployed by plants in response to wounding or pathogen attack have been shown to be induced by certain plant derived chemicals such as methyl jasmonate (MeJA) and salicylic acid (SA). In order to examine changes in the levels of PERK1 mRNA abundance in response to exogenous application of MeJA, *B. napus* plants were thoroughly sprayed with a 50μM MeJA solution. Leaf and stem tissue was subsequently harvested at various times and the steady state levels of PERK1 mRNA were analysed. Figure 5A shows the RNA gel blot and corrected PERK1 mRNA profile for treated leaf tissue during which no significant accumulation of PERK1 mRNA was detected. In response to MeJA, levels of PERK1 transcript in leaf tissue were very weak resembling basal levels in untreated tissue (Figure 3A). Exogenous application of MeJA to stem tissue had no effect on the accumulation of PERK1 mRNA as shown by the corrected profile in which the fold induction of PERK1 did not deviate substantially from the untreated control (0 hr) (Figure 5B). Furthermore, no increase in the steady state levels of PERK1 mRNA was detected in the appropriate control treatment (0.1% [$^{v}/_{v}$] ethanol, solvent control for MeJA) at time 0 hr.

Despite the well established involvement of MeJA (the methyl ester of the plant growth regulator jasmonic acid (JA)) in the signal transduction pathway regulating gene activation upon wounding, processes occurring immediately after wounding remain poorly characterized in terms of additional components that may also participate in wound signaling (Titarenko et al., 1997). Steady state levels of PERK1 mRNA remain unaffected to exogenously applied MeJA (Figure 5) which suggests that the inducibility of PERK1 by wounding occurs via a MeJA-independent pathway (Figue 7). Studies conducted by Titarenko et al. (1997) addressing the role of JA in mediating wound responses support the existence of multiple distinct wound signal transduction pathways. Exogenously applied JA was able to induce only a subset of wound responsive genes in Arabidopsis which ultimately resulted in a stronger systemic accumulation in wounded plants. Conversely, a second set of wound responsive genes showing a stronger induction locally in wounded tissue showed no substantial accumulation upon JA treatment. In conjunction with the pattern of PERK1 mRNA accumulation in response to wounding and MeJA, it appears that plants respond to wounding by two distinct wound signal transduction pathways: one which does not require JA and is primarily responsible for gene activation in the vicinity of the wound site and the other which involves JA perception and most likely activates gene expression both locally and systemically to the wound site (Titarenko et al., 1997).

Many genes isolated to date that are induced by a pathogenic stimulus can be at least partially induced by SA (Ward et al., 1991). In order to address the potential role of PERK1 in a plant's defense response against pathogen attack, 4mM SA was used as a chemical elicitor and sprayed onto *B. napus* plants. Figure 6A shows that when SA is exogenously applied to leaf tissue, PERK1 mRNA accumulates 15 min following treatment reaching a maximum 5 fold induction 45 min post-treatment. Steady state levels of PERK1 mRNA in treated stem tissue peaked at 45 min corresponding to an approximate 2 fold induction in response to 4mM SA (Figure 6B).

Collectively, the profiles of PERK1 mRNA accumulation in response to wounding, MeJA and SA are not entirely surprising. PERK1 induction is rapid in response to wounding (Figure 4) and the lack of PERK1 transcript accumulation in response to MeJA (Figure 5) suggests a pathway for wound mediated induction of PERK1 that is independent of MeJA (Figure 7). The pronounced and rapid induction of PERK1 in response to exogenous SA (Figure 6) supports other studies showing that SA is known to inhibit wound responsive genes that are regulated by a MeJA-dependent pathway (Peña-Cortés et al., 1993; Doares et al., 1995). Therefore, it is unlikely that both MeJA and SA would induce PERK1 mRNA accumulation given that these pathways are known to be antagonistic (Peña-Cortés et al., 1993). Nevertheless, the rapid induction of PERK1 during these treatment suggests a potential role early on in a plant's defense signaling pathway.

Protein work on PERK1

The generation of a PERK1 antibody would be an invaluable tool in studies to address the expression, localization, regulation and potential function of PERK1 of the present invention. The coding sequence for the region containing the extracellular domain of PERK1 of the present invention as well as the coding sequence representing the entire protein were cloned into the pTricHis expression plasmid for production of His-tagged fusion proteins in *E. coli* and

purification by affinity chromatography on a Talon resin column.

Figure 8A represents a Western blot performed on the bacterially expressed extracellular domain fusion protein using a T7 monoclonal antibody which recognizes the T7 epitope on the

bacterially expressed fusion protein. There was a marked induction of the fusion protein in the presence of IPTG (Isopropyl-β-d-Thiogalactopyranoside) (Figure 8A; lane 2), however recovery of the purified protein (Lane 4 ; ♦) was not very efficient. The presence of the fusion protein in the insoluble fraction (Lane 3) suggests that the extracellular domain is likely insoluble and could be purified from inclusion bodies. This method relies on the bacterial expression system generating large amounts of the insoluble fusion protein which form inclusion bodies in the bacterial host. Figure 8B is a Western blot to confirm that the extracellular domain fusion protein is targeted to inclusion bodies. Induction of the fusion protein was efficient (Figure 8B; lane2), and despite its presence in the supernatant (Lane 3) and in the denatured insoluble samples (Lane 5), the fusion protein is predominantly in the insoluble fraction (IB-inclusion body) (Lane 4 ; ◊) Lanes 6-9 represent the pTric HisC negative control indicating that the signals obtained for the expression of the extracellular domain protein are specific to the fusion protein. The extracellular domain fusion protein can be used as an antigen and injected into rabbits in order to generate a polyclonal antibody against the extracellular domain of PERK1. This fusion protein can either be injected directly as an inclusion body in the presence of an adjuvant, or alternatively, the protein purified from the inclusion bodies can be separated on an SDS-Polyacrylamide gel, and the region of the gel at which the protein of interest migrates can be excised and used for injection. It should be noted that the bacterially expressed fusion protein is migrating at a higher molecular weight than predicted from the amino acid sequence, however this is not entirely uncommon and could in fact be due to the high percentage of proline residues (40%) comprising the extracellular domain of PERK1.

Similar experiments were conducted for the fusion protein corresponding to the entire coding region of PERK1. Figure 9 is a Western blot conducted on the bacterially expressed PERK1 protein to determine whether the full length protein is insoluble and forms inclusion bodies. Although the induction of the full length protein was not as pronounced (Figure 9; lane 2) it is quite evident that the fusion protein is predominantly localized in the insoluble fraction (i.e. inclusion body) (Lane 4; ■) Lanes 6-9 represent the parental plasmid as mentioned above. We are currently modifying several variables of the induction protocol in an attempt to maximize the yield of full length PERK1 protein. Once these conditions are optimized, the PERK1 fusion

protein can also be used as an antigen to raise antibody against full length PERK1 as previously described.

In order to determine whether PERK1 encodes a protein with kinase activity as its sequence predicts, the bacterially expressed catalytic domain fusion protein of PERK1 was tested for kinase activity. Furthermore, to ensure that the phosphorylation of the fusion protein was not a result of bacterial kinase activity, a mutated catalytic domain was also generated by site directed mutagenesis which introduced a single base pair substitution of a lysine residue to a glutamic acid residue (L →E). This mutation modifies the essential invariant lysine of subdomain II required for phospho-transfer and renders the kinase inactive. Both the wild-type and the mutated catalytic domains of PERK1 were cloned into the pMAL-c expression system, induced for protein production in the presence of IPTG and purified by affinity chromatography on MBP amylose resin. Figure 10A is a Western blot to confirm the induction and purification of both the wild-type (★) and mutated (◀) catalytic domain fusion proteins using an anti-MBP antibody. The wild-type fusion protein appears to be toxic in bacteria which compromises its inducibility and purification (Figure 10A; lanes 3-4). In addition, the wild-type fusion protein migrates at a slightly larger molecular weight than does the mutated protein. The mutated fusion protein is induced and purified more efficiently, perhaps due to the fact that it is no longer kinase active (Figure 10A ; Lanes 6-7). Purified pMAL-c (Lane 1; •) was used as a negative control for this experiment.

Figure 10B represents a kinase assay performed on affinity purified wild-type and mutated fusion proteins incubated in the presence of γ-^{32}PdATP. Detection of a phosphoprotein only in Lane 1 provides direct biochemical evidence that the wild-type catalytic domain (★) of PERK1 encodes a functional protein kinase that is capable of autophosphorylation (Lane 1) and that the mutation successfully abolished kinase activity (Lane 2).

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Although the invention has been described with reference to certain specific embodiments, various modifications thereof will be apparent to those skilled in the art without departing from the spirit and scope of the invention as outlined in the claims appended hereto.

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. An isolated DNA molecule comprising the nucleic acid sequence of SEQ ID No 1.
2. An isolated DNA molecule having at least 30% homology to the nucleic acid sequence of SEQ ID No 1.
3. An amino acid molecule having the amino acid of SEQ ID No 2.
4. An isolated DNA molecule coding for the amino acid sequence of claim 3.
5. A cell transfected with the nucleic acid sequence of SEQ ID No 1.

ABSTRACT

An isolated receptor-like protein kinase designated PERK1 (Proline Extensin-like Receptor Kinase), isolated from the cDNA library of *Brassica napus*, represents a new class of plant receptor kinases characterized by an extracellular domain rich in proline and sharing sequence similarity to the extensin family of cell wall proteins. Also provided is a protein expressed by the isolated nucleic acid molecule as well as cell lines transfected with the same.

A.

```
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                                                           S  A  P  S  P  G
 25 ACTGGTTCGCCTCCATCTCCACCATCAAACTCCACAACCACCACTCCTCCTCCAGCTTCC            84
    T  G  S  P  P  S  P  P  S  N*  S  T  T  T  T  P  P  P  A  S
 85 GCTCCTCCTCCCACCACACCTTCTTCTCCTCCGCCGCCATCCACTATTCCGACATCTCCT           144
    A  P  P  P  T  T  P  S  S  P  P  P  P  S  T  I  P  T  S  P
145 CCTCCTTCTTCTCGCTCTACACCTTCTGCTCCTCCTCCATCTCCACCAACTCCATCTACG           204
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445 CTGCTTGTGATAGTGACTCTGATTTGTCTCCTCTGTAAGAAGAAACGACGGAGAGACGAA           504
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    S  G  K  L  T  E  K  S  D  V  F  S  F  G  V  V  L  L  E  L
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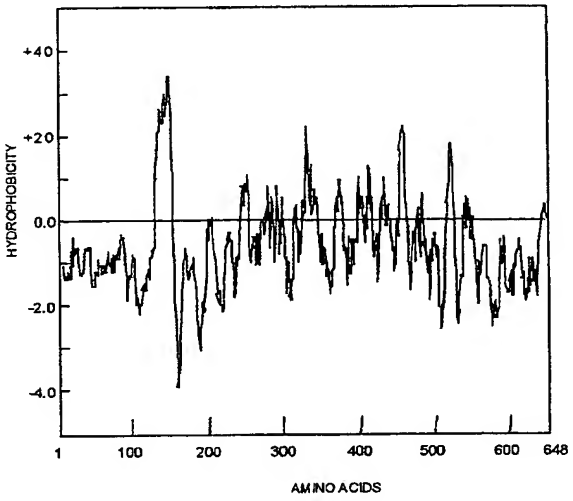

Figure1

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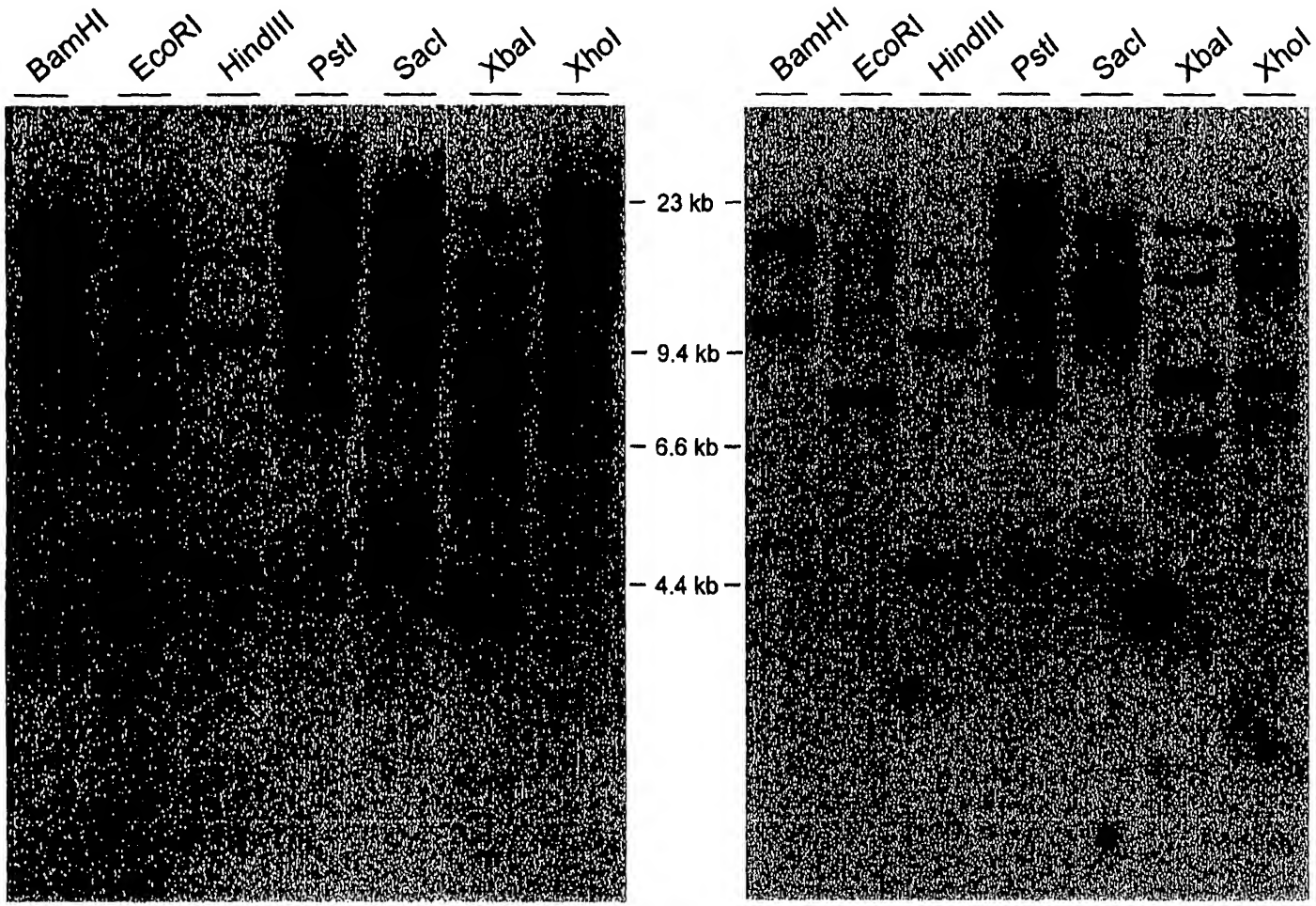
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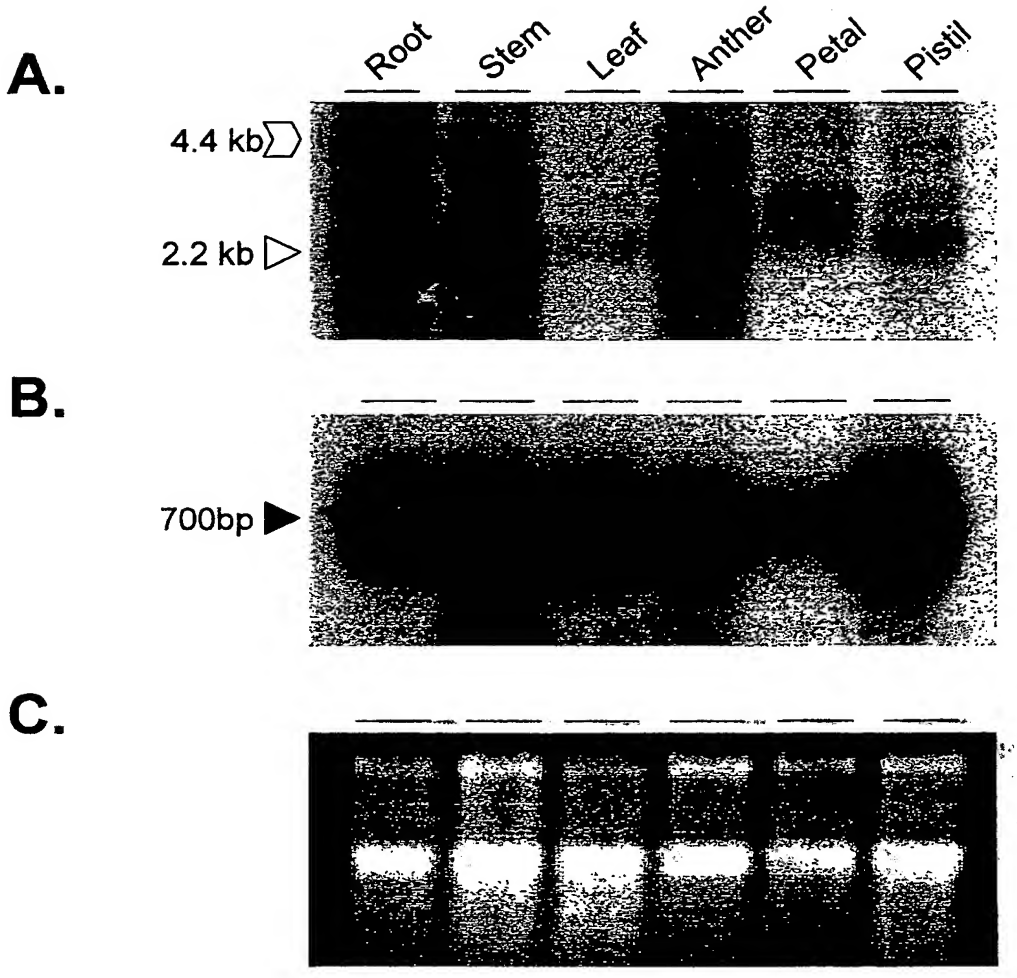
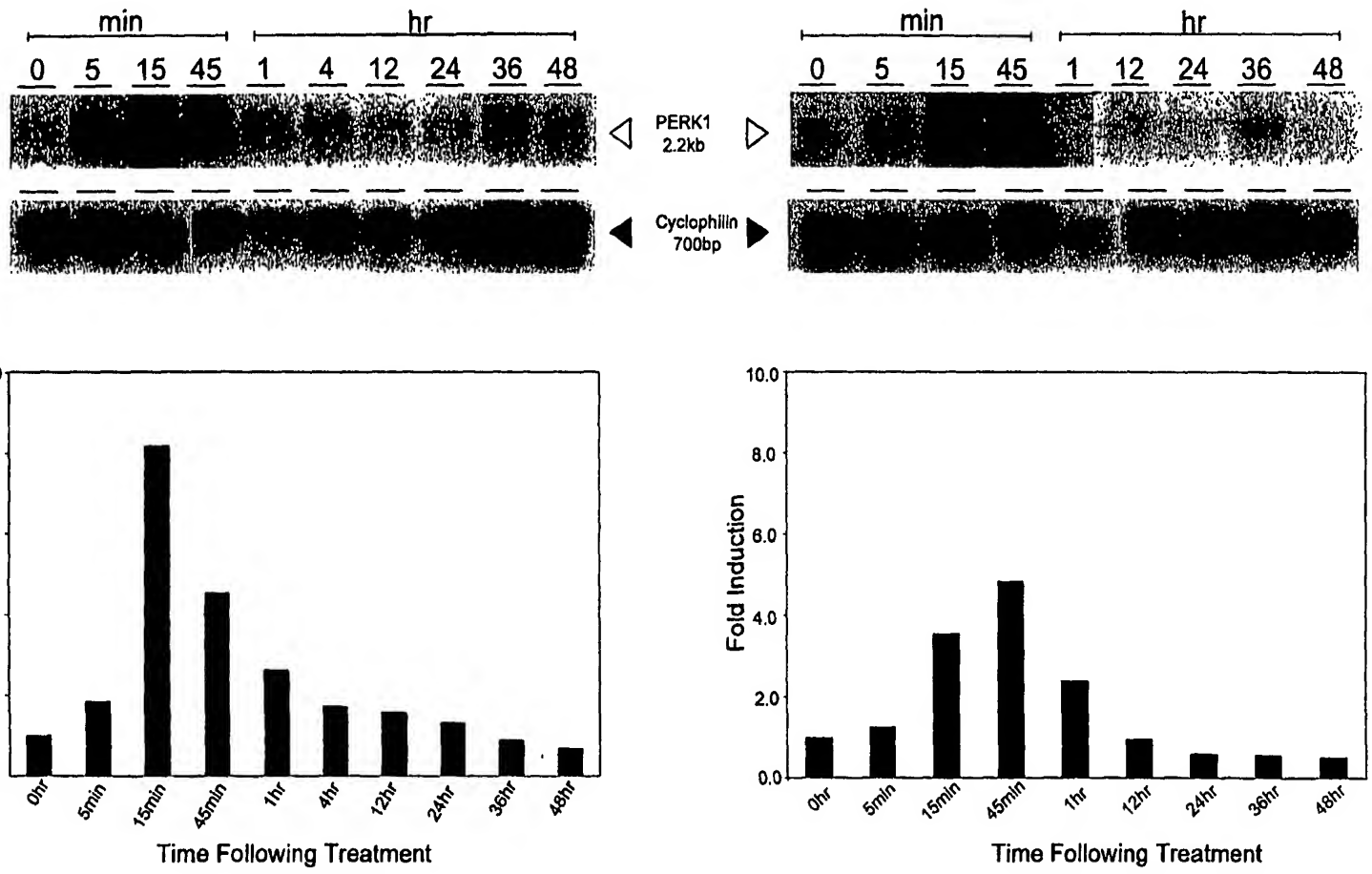


Figure 3

Figure 4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Fold Induction
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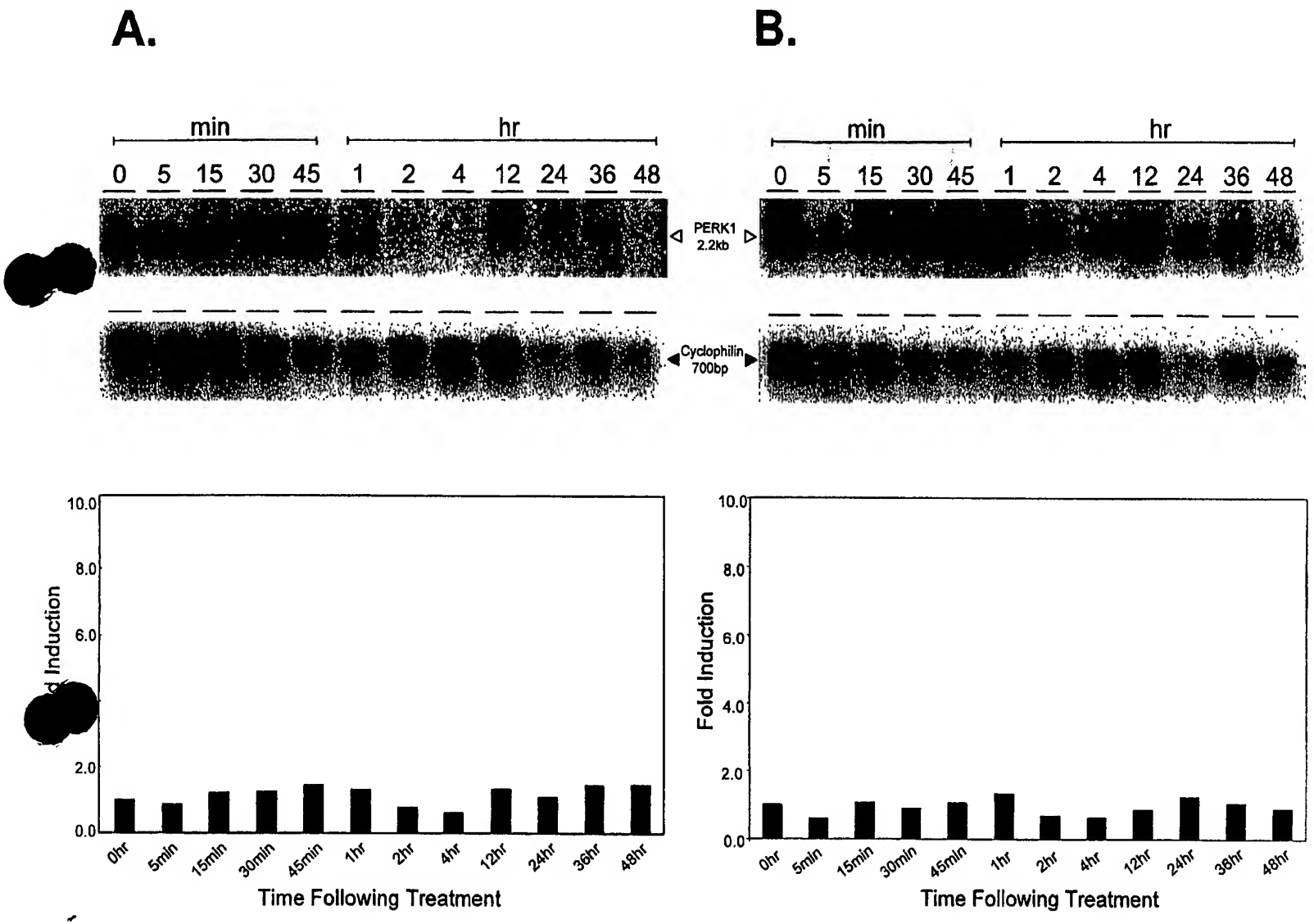
Time Following Treatment

Figure 5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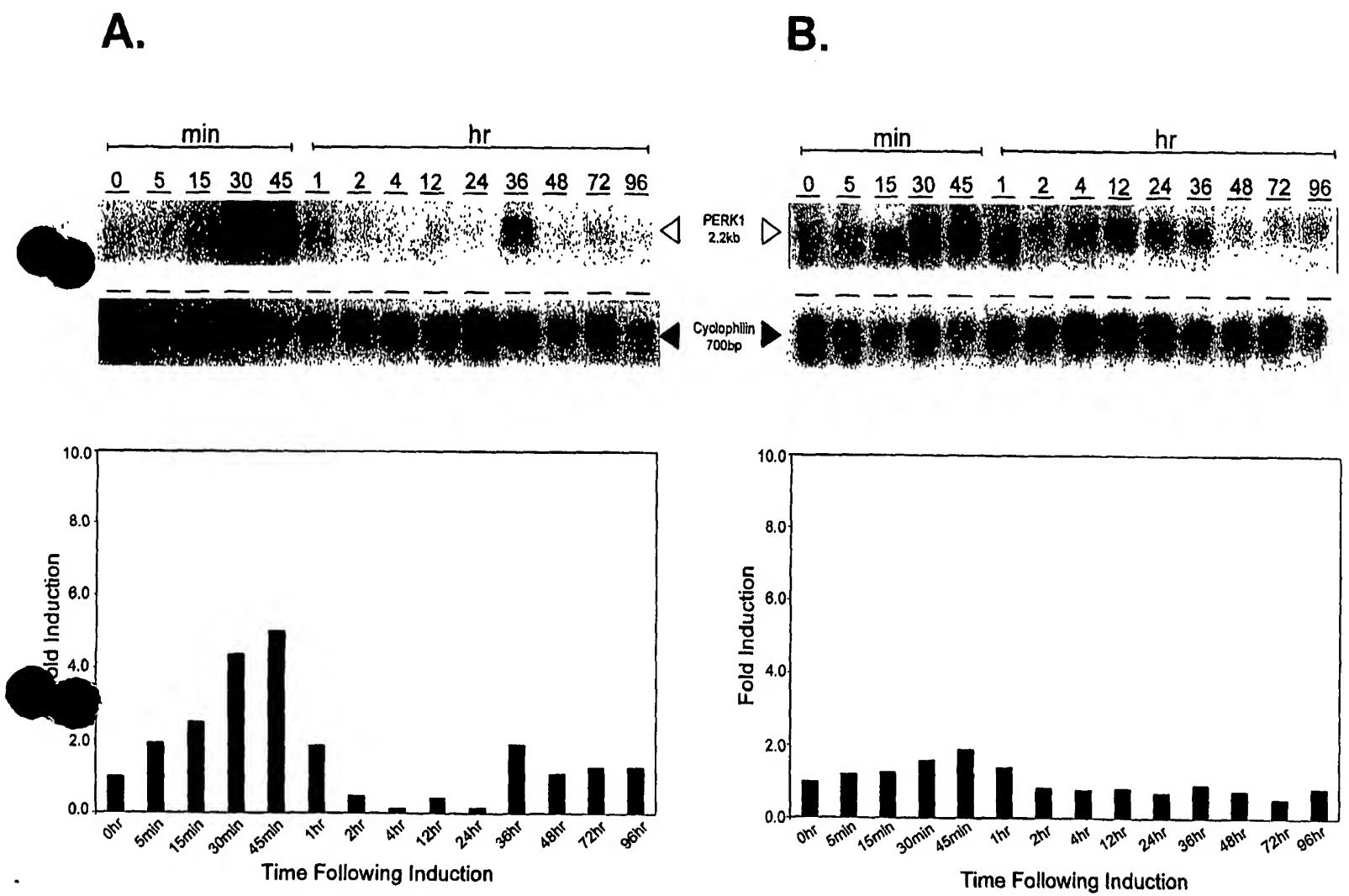
Time Following Treatment

Figure 6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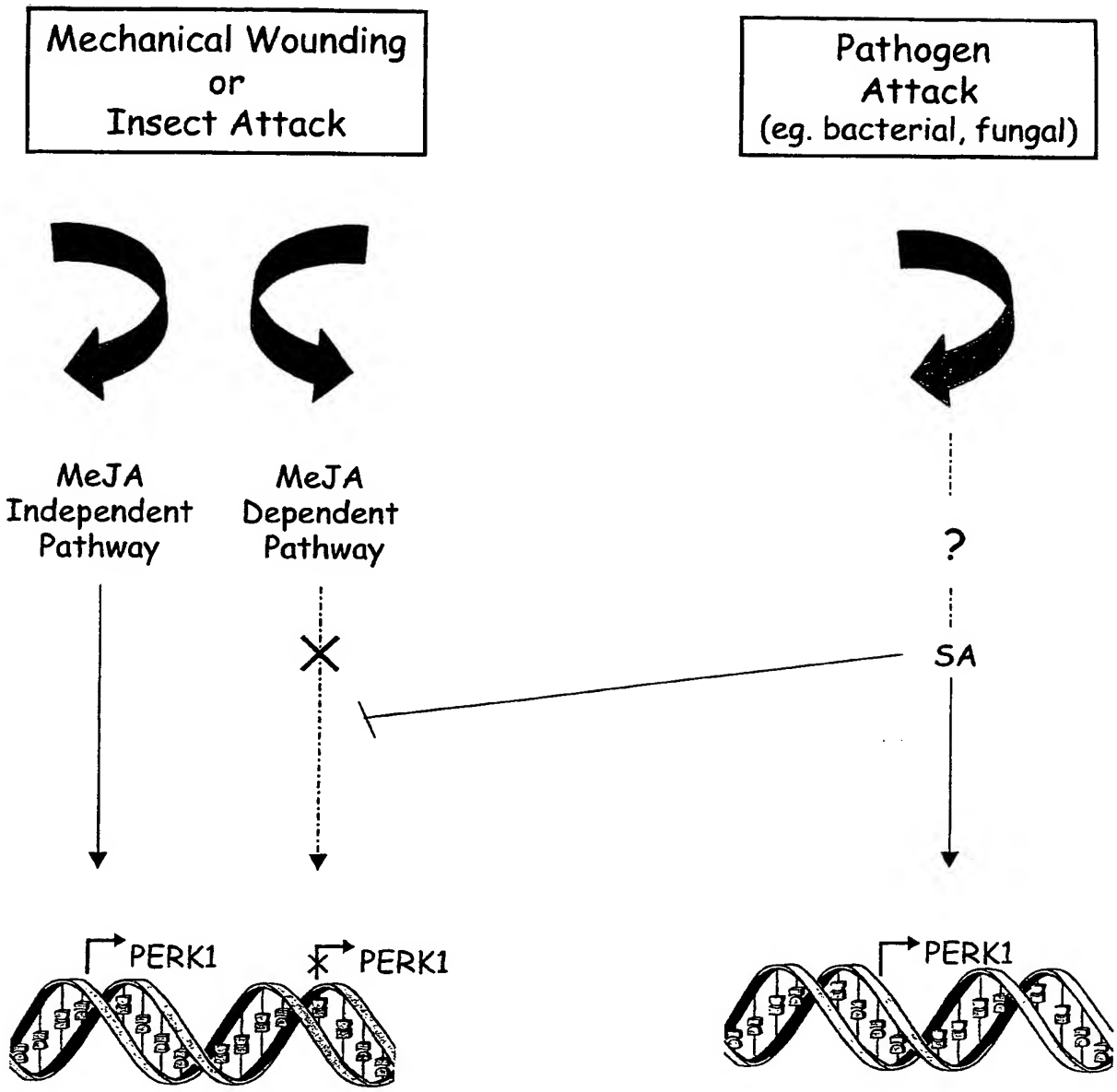
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or
Insect Attack
Pathogen
Attack
(eg. bacterial, fungal)
MeJA
Independent
Pathway
MeJA
Dependent
Pathway
?
SA
PERK1
PERK1
PERK1

Figure 7

Bacterially Expressed Extracellular Domain of PERK1

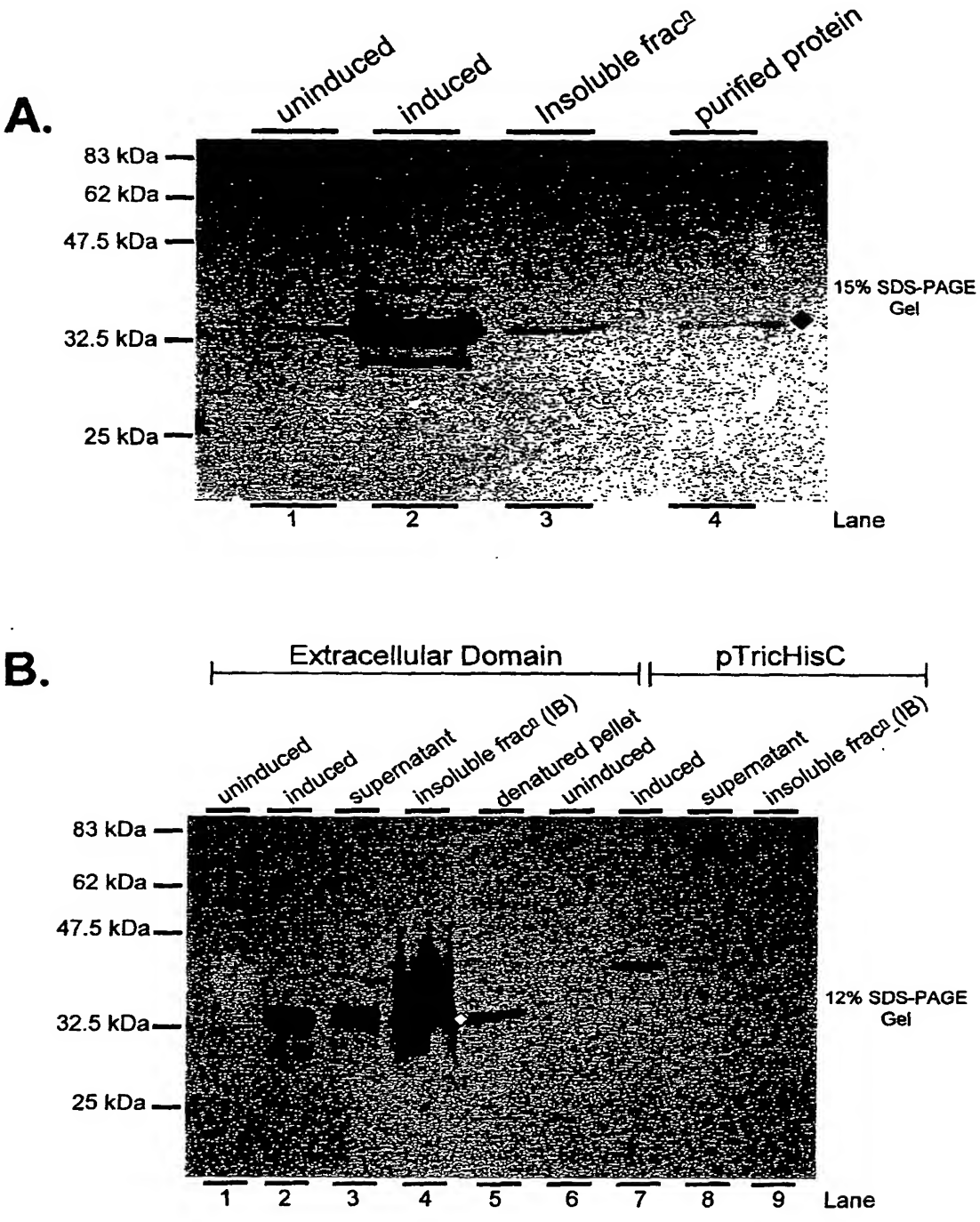


Figure 8

Bacterially Expressed Full Length PERK1

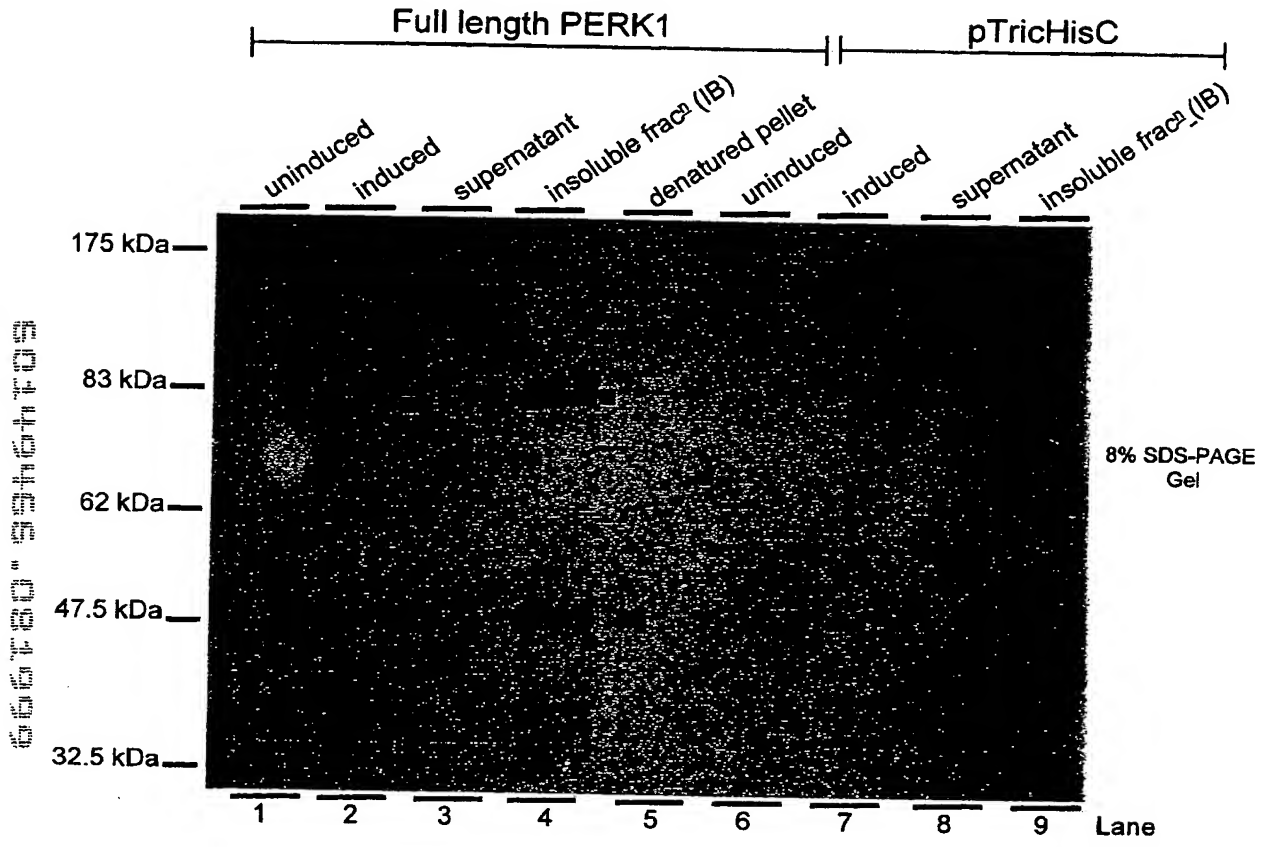


Figure 9

Bacterial Expres[redacted]n and Kinase Assay of Wild-Typ[redacted]d Mutated Catalytic Domains of PERK1

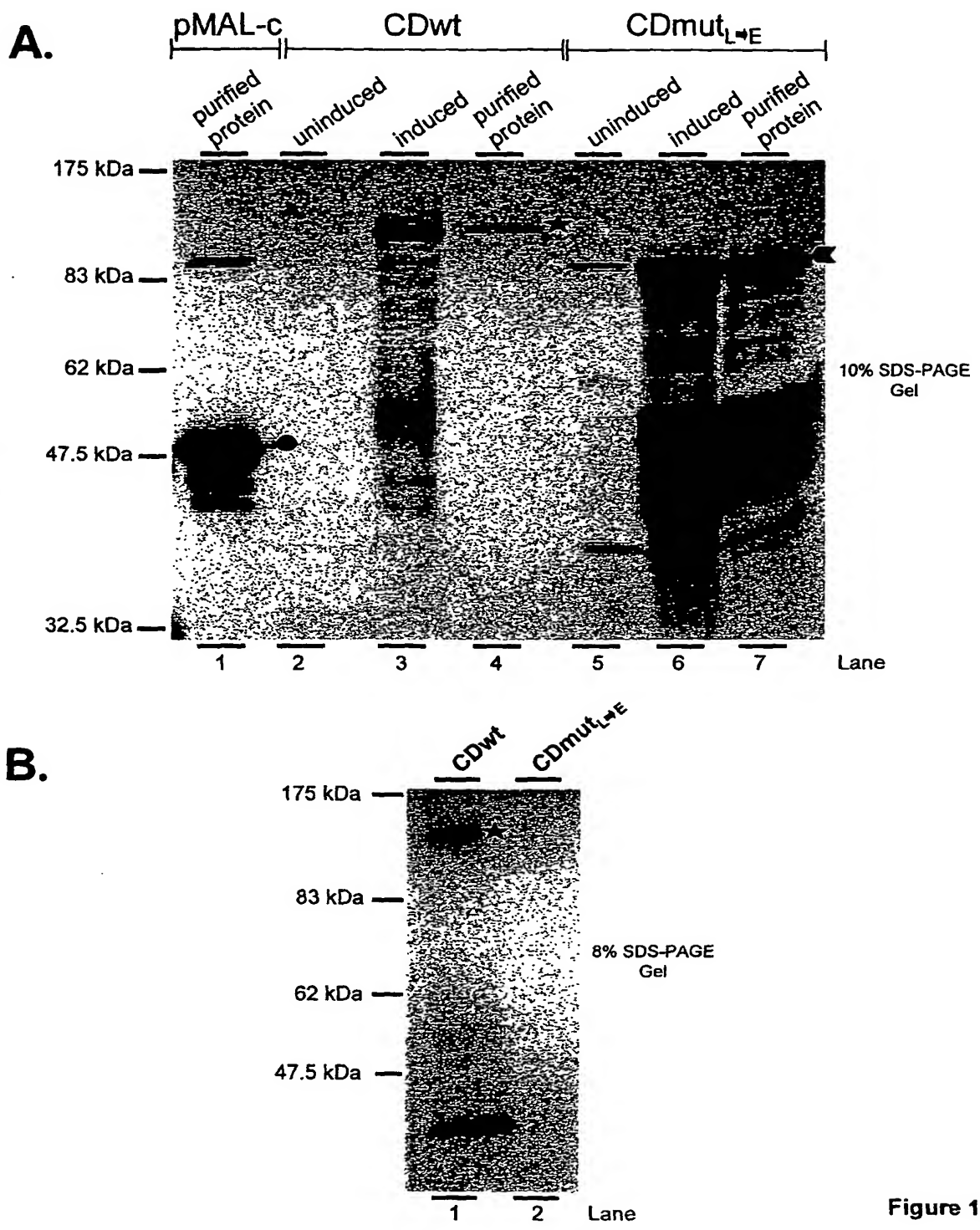
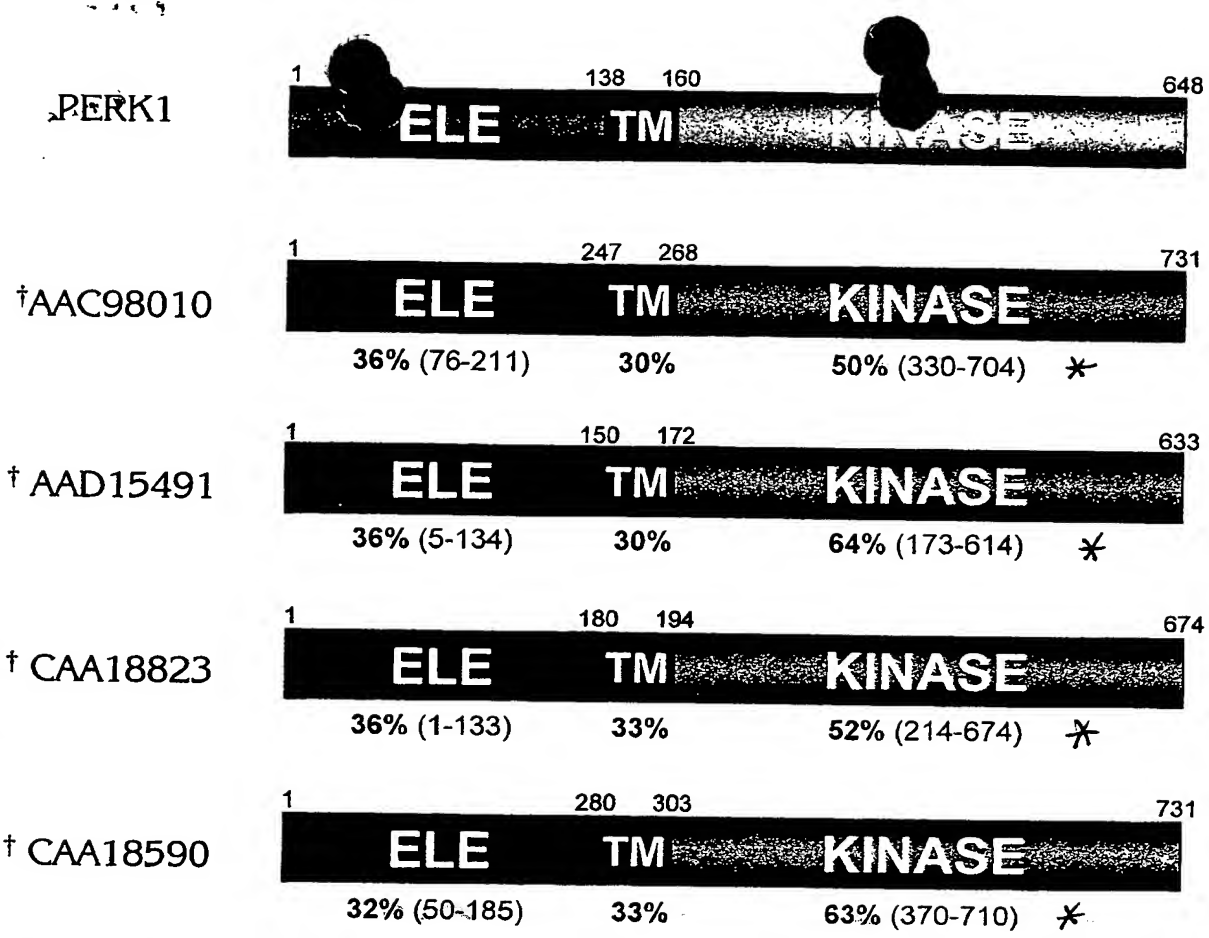


Figure 10



All have no signal peptide and predicted to be Type1b integral membrane proteins

† Predicted proteins from the Arabidopsis genome sequencing project.

ELE: Extensin-like extracellular ; TM: Transmembrane domain ; * Red = sequence identity to PERK1 domain

	PERK1	ACC98010	AAD15491	CAA18823	CAA18590
PERK1		50%	64%	52%	63%
ACC98010	36%		54%	50%	52%
AAD15491	36%	25%		63%	51%
CAA18823	36%	29%	27%		60%
CAA18590	32%	36%	30%	27%	

Kinase Domains

ELE Domains

FIGURE 11

SEQUENCE LISTING

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Silva, Nancy F.
Mazzurco, Maria

<120> Characterization Of A Novel Receptor Kinase From Brassica With A Putative Role In Plant Defense

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Met Ser Ser Ala Pro Ser
1 5

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Pro Gly Thr Gly Ser Pro Pro Ser Pro Pro Ser Asn Ser Thr Thr Thr
10 15 20

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Thr Pro Pro Pro Ala Ser Ala Pro Pro Pro Thr Thr Pro Ser Ser Pro
25 30 35

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Pro Pro Pro Ser Thr Ile Pro Thr Ser Pro Pro Pro Ser Ser Arg Ser
40 45 50

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[redacted] [redacted]

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Thr Pro Ser Ala Pro Pro Pr  Ser Pro Pro Thr Pro Ser Thr Pro Gly
 55                  60                  65                  70

tct cca cct cct ctt cct cag ccg tct cca ccc gct cca act acg ccc   354
Ser Pro Pro Pro Leu Pro Gln Pro Ser Pro Pro Ala Pro Thr Thr Pro
                 75                  80                  85

gga tct cca ccc gca cct gtt act cct cct act cga aac cct cca cct   402
Gly Ser Pro Pro Ala Pro Val Thr Pro Pro Thr Arg Asn Pro Pro Pro
             90                  95                 100

tca gtc cca gga cca ccg tcc aat cct tca cgc gaa gga gga tct cct   450
Ser Val Pro Gly Pro Pro Ser Asn Pro Ser Arg Glu Gly Gly Ser Pro
        105                 110                 115

cga cct cca tct tct ccc tcg ccg ccg tct cct tct tcc gac ggt tta   498
Arg Pro Pro Ser Ser Pro Ser Pro Pro Ser Pro Ser Ser Asp Gly Leu
    120                 125                 130

tca aca gga gtg gtg gtg gga atc gcc atc gga gga gtc gct ctg ctt   546
Ser Thr Gly Val Val Val Gly Ile Ala Ile Gly Gly Val Ala Leu Leu
135                 140                 145                 150

gtg ata gtg act ctg att tgt ctc ctc tgt aag aag aaa cga cgg aga   594
Val Ile Val Thr Leu Ile Cys Leu Leu Cys Lys Lys Lys Arg Arg Arg
                155                 160                 165

gac gaa gaa gat gct tac tat gtt cct ccg cca cct cct cct ggt ccc   642
Asp Glu Glu Asp Ala Tyr Tyr Val Pro Pro Pro Pro Pro Pro Gly Pro
            170                 175                 180

aaa gcc gga gga cct tac ggt gga cag cag caa caa tgg cgg caa caa   690
Lys Ala Gly Gly Pro Tyr Gly Gly Gln Gln Gln Gln Trp Arg Gln Gln
        185                 190                 195

aac gca aca cca ccg tca gat cat gtc gtg acg tca cta cca cca cca   738
Asn Ala Thr Pro Pro Ser Asp His Val Val Thr Ser Leu Pro Pro Pro
    200                 205                 210

cct aag gct cca tct cca cca cgg caa cct cct cca cct cca cca ccg   786
Pro Lys Ala Pro Ser Pro Pro Arg Gln Pro Pro Pro Pro Pro Pro Pro
215                 220                 225                 230

cct ttc atg agc agc agc ggc ggc tcc gac tac tcg gac cgt cca gtt   834
Pro Phe Met Ser Ser Ser Gly Gly Ser Asp Tyr Ser Asp Arg Pro Val
                235                 240                 245

ctt cct cca ccg tct cca ggg ctt gtg tta ggc ttc tcc aaa agc act   882
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L u Pro Pro Pro Ser Pro Gly Leu Val Leu Gly Phe Ser Lys S r Thr
            250                 255                 260

ttc aca tac gag gag cta gct aga gcc acc aat ggt ttc tcc gag gcg   930
Phe Thr Tyr Glu Glu Leu Ala Arg Ala Thr Asn Gly Phe Ser Glu Ala
        265                 270                 275

aac ttg tta gga caa ggc ggg ttc ggt tac gtg cac aaa ggt gtg ttg   978
Asn Leu Leu Gly Gln Gly Gly Phe Gly Tyr Val His Lys Gly Val Leu
    280                 285                 290

cct agt ggg aaa gaa gtt gct gtg aag cag ttg aaa gtt ggg agt ggt   1026
Pro Ser Gly Lys Glu Val Ala Val Lys Gln Leu Lys Val Gly Ser Gly
295                 300                 305                 310

cag gga gag agg gag ttt cag gca gag gtt gag atc atc agc aga gtt   1074
Gln Gly Glu Arg Glu Phe Gln Ala Glu Val Glu Ile Ile Ser Arg Val
                315                 320                 325

cac cac agg cat ctg gtg tct ctt gtt ggt tat tgc atc gcc ggt gcc   1122
His His Arg His Leu Val Ser Leu Val Gly Tyr Cys Ile Ala Gly Ala
            330                 335                 340

aaa aga ttg ctt gtc tat gag ttt gtt cct aac aac aat ctc gag ctt   1170
Lys Arg Leu Leu Val Tyr Glu Phe Val Pro Asn Asn Asn Leu Glu Leu
        345                 350                 355

cac ctc cat ggc gag gga cgg cct aca atg gaa tgg agc acc aga ttg   1218
His Leu His Gly Glu Gly Arg Pro Thr Met Glu Trp Ser Thr Arg Leu
    360                 365                 370

aag att gct ctt gga tct gct aaa gga ctt tct tat ctt cat gaa gat   1266
Lys Ile Ala Leu Gly Ser Ala Lys Gly Leu Ser Tyr Leu His Glu Asp
375                 380                 385                 390

tgc aat cct aaa atc att cac cgt gat atc aag gct tca aac ata ttg   1314
Cys Asn Pro Lys Ile Ile His Arg Asp Ile Lys Ala Ser Asn Ile Leu
                395                 400                 405

ata gat ttc aag ttt gaa gct aag gtt gct gat ttt ggt ctt gct aag   1362
Ile Asp Phe Lys Phe Glu Ala Lys Val Ala Asp Phe Gly Leu Ala Lys
            410                 415                 420

att gct tct gat aca aac acg cat gta tca aca cgt gtg atg gga acc   1410
Ile Ala Ser Asp Thr Asn Thr His Val Ser Thr Arg Val Met Gly Thr
        425                 430                 435

ttt ggg tac ttg gct ccg gaa tac gct gca agc gga aag ctc acg gag   1458
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Phe Gly Tyr Leu Ala Pro Glu Tyr Ala Ala Ser Gly Lys Leu Thr Glu
    440                 445                 450

aag tct gac gtt ttc tca ttt ggc gtt gtg ctt ttg gag ctc att act   1506
Lys Ser Asp Val Phe Ser Phe Gly Val Val Leu Leu Glu Leu Ile Thr
455                 460                 465                 470

gga cgt cga ccc gtt gat gcc aac aat gtc tat gta gat gac agc tta   1554
Gly Arg Arg Pro Val Asp Ala Asn Asn Val Tyr Val Asp Asp Ser Leu
                475                 480                 485

gtt gac tgg gca cga cca ttg ctt aac cga gca tct gag caa gga gac   1602
Val Asp Trp Ala Arg Pro Leu Leu Asn Arg Ala Ser Glu Gln Gly Asp
            490                 495                 500

ttt gag ggt tta gct gat gca aag atg aat aat ggg tat gac aga gag   1650
Phe Glu Gly Leu Ala Asp Ala Lys Met Asn Asn Gly Tyr Asp Arg Glu
        505                 510                 515

gag atg gct cgc atg gtt gct tgt gct gcg gct tgt gtt cgc cat tca   1698
Glu Met Ala Arg Met Val Ala Cys Ala Ala Ala Cys Val Arg His Ser
    520                 525                 530

gct cgc cgc aga cct cgc atg agc cag att gtg cgt gcg tta gaa gga   1746
Ala Arg Arg Arg Pro Arg Met Ser Gln Ile Val Arg Ala Leu Glu Gly
535                 540                 545                 550

aat gta tca ctg tca gat ctt aac gaa ggg atg aga cca ggt caa agc   1794
Asn Val Ser Leu Ser Asp Leu Asn Glu Gly Met Arg Pro Gly Gln Ser
                555                 560                 565

aat gta tac agc tca tac gga gga agc acc gat tat gac tcg agc cag   1842
Asn Val Tyr Ser Ser Tyr Gly Gly Ser Thr Asp Tyr Asp Ser Ser Gln
            570                 575                 580

tac aat gaa gac atg aag aag ttt agg aaa atg gca ctt gga act caa   1890
Tyr Asn Glu Asp Met Lys Lys Phe Arg Lys Met Ala Leu Gly Thr Gln
        585                 590                 595

gag tac aac gcc acg ggt gag tac agt aat ccg acc agt gac tat gga   1938
Glu Tyr Asn Ala Thr Gly Glu Tyr Ser Asn Pro Thr Ser Asp Tyr Gly
    600                 605                 610

ctg tac ccg tct ggt tca agc agc gag ggc caa acc aca cgc gaa atg   1986
Leu Tyr Pro Ser Gly Ser Ser Ser Glu Gly Gln Thr Thr Arg Glu Met
615                 620                 625                 630

gag atg ggg aag att aag aga acc ggt cag ggt tat agt gga cct tct   2034
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Glu M t Gly Lys Ile Lys Arg Thr Gly Gln Gly Tyr Ser Gly Pro Ser
635 640 645

ctt taa accagatggg agagaaattg aagggtgttt tttcattatt tttttaaaac 2090
Leu

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tacgttttga attggttttt aaaaaaaaaa aaaaaaaaa 2189

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Ser Asn Ser Thr Thr Thr Thr Pro Pro Pro Ala Ser Ala Pro Pro Pro
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Thr Thr Pro Ser Ser Pro Pro Pro Pro Ser Thr Ile Pro Thr Ser Pro
35 40 45

Pro Pro Ser Ser Arg Ser Thr Pro Ser Ala Pro Pro Pro Ser Pro Pro
50 55 60

Thr Pro Ser Thr Pro Gly Ser Pro Pro Pro Leu Pro Gln Pro Ser Pro
65 70 75 80

Pro Ala Pro Thr Thr Pro Gly Ser Pro Pro Ala Pro Val Thr Pro Pro
85 90 95

Thr Arg Asn Pro Pro Pro Ser Val Pro Gly Pro Pro Ser Asn Pro Ser
100 105 110

Arg Glu Gly Gly Ser Pro Arg Pro Pro Ser Ser Pro Ser Pro Pro Ser
115 120 125

Pro Ser Ser Asp Gly Leu Ser Thr Gly Val Val Val Gly Ile Ala Ile
130 135 140

Gly Gly Val Ala Leu Leu Val Ile Val Thr Leu Ile Cys Leu Leu Cys
145 150 155 160

Lys Lys Lys Arg Arg Arg Asp Glu Glu Asp Ala Tyr Tyr Val Pro Pro

[redacted] [redacted]

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                165                 170                 175

Pro Pro Pro Pro Gly Pro Lys Ala Gly Gly Pro Tyr Gly Gly Gln Gln
            180                 185                 190

Gln Gln Trp Arg Gln Gln Asn Ala Thr Pro Pro Ser Asp His Val Val
        195                 200                 205

Thr Ser Leu Pro Pro Pro Pro Lys Ala Pro Ser Pro Pro Arg Gln Pro
    210                 215                 220

Pro Pro Pro Pro Pro Pro Pro Phe Met Ser Ser Ser Gly Gly Ser Asp
225                 230                 235                 240

Tyr Ser Asp Arg Pro Val Leu Pro Pro Pro Ser Pro Gly Leu Val Leu
                245                 250                 255

Gly Phe Ser Lys Ser Thr Phe Thr Tyr Glu Glu Leu Ala Arg Ala Thr
            260                 265                 270

Asn Gly Phe Ser Glu Ala Asn Leu Leu Gly Gln Gly Gly Phe Gly Tyr
        275                 280                 285

Val His Lys Gly Val Leu Pro Ser Gly Lys Glu Val Ala Val Lys Gln
    290                 295                 300

Leu Lys Val Gly Ser Gly Gln Gly Glu Arg Glu Phe Gln Ala Glu Val
305                 310                 315                 320

Glu Ile Ile Ser Arg Val His His Arg His Leu Val Ser Leu Val Gly
                325                 330                 335

Tyr Cys Ile Ala Gly Ala Lys Arg Leu Leu Val Tyr Glu Phe Val Pro
            340                 345                 350

Asn Asn Asn Leu Glu Leu His Leu His Gly Glu Gly Arg Pro Thr Met
        355                 360                 365

Glu Trp Ser Thr Arg Leu Lys Ile Ala Leu Gly Ser Ala Lys Gly Leu
    370                 375                 380

Ser Tyr Leu His Glu Asp Cys Asn Pro Lys Ile Ile His Arg Asp Ile
385                 390                 395                 400

Lys Ala Ser Asn Ile Leu Ile Asp Phe Lys Phe Glu Ala Lys Val Ala
                405                 410                 415

Asp Phe Gly Leu Ala Lys Ile Ala Ser Asp Thr Asn Thr His Val Ser
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                420                 425                 430

Thr Arg Val Met Gly Thr Phe Gly Tyr Leu Ala Pro Glu Tyr Ala Ala
        435                 440                 445

Ser Gly Lys Leu Thr Glu Lys Ser Asp Val Phe Ser Phe Gly Val Val
    450                 455                 460

Leu Leu Glu Leu Ile Thr Gly Arg Arg Pro Val Asp Ala Asn Asn Val
465                 470                 475                 480

Tyr Val Asp Asp Ser Leu Val Asp Trp Ala Arg Pro Leu Leu Asn Arg
                485                 490                 495

Ala Ser Glu Gln Gly Asp Phe Glu Gly Leu Ala Asp Ala Lys Met Asn
            500                 505                 510

Asn Gly Tyr Asp Arg Glu Glu Met Ala Arg Met Val Ala Cys Ala Ala
        515                 520                 525

Ala Cys Val Arg His Ser Ala Arg Arg Arg Pro Arg Met Ser Gln Ile
    530                 535                 540

Val Arg Ala Leu Glu Gly Asn Val Ser Leu Ser Asp Leu Asn Glu Gly
545                 550                 555                 560

Met Arg Pro Gly Gln Ser Asn Val Tyr Ser Ser Tyr Gly Gly Ser Thr
                565                 570                 575

Asp Tyr Asp Ser Ser Gln Tyr Asn Glu Asp Met Lys Lys Phe Arg Lys
            580                 585                 590

Met Ala Leu Gly Thr Gln Glu Tyr Asn Ala Thr Gly Glu Tyr Ser Asn
        595                 600                 605

Pro Thr Ser Asp Tyr Gly Leu Tyr Pro Ser Gly Ser Ser Ser Glu Gly
    610                 615                 620

Gln Thr Thr Arg Glu Met Glu Met Gly Lys Ile Lys Arg Thr Gly Gln
625                 630                 635                 640

Gly Tyr Ser Gly Pro Ser Leu
                645
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